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13. ABSTRACT (Maximum 200 words)

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ppb was found to be 70 to 99%; (4) Immunochromatography has been shown to be an effective method for identification of MCYST-LR and related MCYSTs in liver cytosol, feces and algal blooms; (5) Immunoaffinity chromatographic methods were established for purification of MCYST and STX; affinity chromatography method was also established for the purification of antibodies against MCYST and STX; (6) Critical evaluation of established immunoassays was made through analysis of various naturally-occurring MCYST containing samples. We found that the ELISA could have a wide application including water supplies, algal samples, lake sediment, phytoplankton, and animal tissue and organs, as well as urine and serum. The pure culture of toxic _Microcystis_ contained about 4-7 mg of MCYST per gram of dry cells. Microcystin accumulated in the hepatopancreas of toxic clams rather than the soft tissues. Microcystin could also be detected by ELISA in naturally-occurring algae bloom samples that contained _Anabaena_ and _Oscillatoria_. (7) Analysis of lake water containing MCYST before and after municipal water treatment revealed that more than 80% of MCYST in the lake water at the level of 17.3 to 338 pg/mL (ppt) was removed; (8) Several approaches were found to be very effective in improving the immunoassay for MCYST and STX. This included using affinity purified marker ligands and antibodies as well as coating a toxin-protein conjugate with less affinity to the antibodies; (9) ELISA analysis of the kinetics of distribution of the MCYST-LR in serum and liver cytosol of mice dosed with radioactive toxin revealed that the toxin peaked in serum and cytosol at 2 hr and 12 hr, respectively. MCYST was still detectable 24 hrs post-dosing. An excellent correlation ($r=0.99$ at $p < 0.0001$) between data obtained from ELISA and radiotracer methods was found. (10) Both Mab and Pab have shown to reverse the inhibitory effect of MCYST to protein phosphatase (PP) 2A activity, but Mab was less effective. The antibodies didn't reverse the inhibitory effect caused by okadaic acid and calyculin A; (11) Anti-idiotype antibodies against rabbit anti-MCYST Pab were demonstrated in mice. Several hybridoma cell lines producing the anti-MCYST anti-idiotype antibodies were obtained; (12) Several new immunogens, including hemiglutarate of decarbamoylated saxitoxin (DC-STX) conjugated to bovine serum albumin (BSA) and to keyhole limpet hemocyanin (KLH), carboxylmethoxyl derivative of STX (CMO) to BSA were prepared and their efficacy for antibody production were examined. These immunogens were not as effective as those prepared by Mannisch reaction where formaldehyde was used as the cross-linking reagent; (13) Pabs against neo-STX were produced and characterized. The antibodies not only are specific for neo-STX, but also have good cross-reactivity with STX. The ID $_{50}$ values for the binding of neo-STX-BSA to the anti-neo-STX-KLH by neo-STX, STX and DC-STX were 0.9, 8.0 and 53.1 ng/mL, respectively. However, the ID $_{50}$ values for the binding of anti-neo-STX-KLH to STX-polylysine coated on the microtiter plate by neo-STX, STX, and DC-STX were 1.2, 4.1 and 36.1 ng/mL, respectively. Using these antibodies, an effective ELISA for the determination of both STX and neo-STX was established; (14) Several hybridoma cell lines producing Mab against neo-STX were obtained. However, the affinity Mab to neo-STX and STX was much lower than Pab; (15) An improved procedure, including the use of immunoaffinity chromatography, for the isolation of STX and neo-STX from algal cells was established; (16) Five ml of anti-MCYST antiserum were delivered.

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FOREWORD

The following is the midterm report of the work performed under contract No. DAMD17-90-C-0002 during the period of Nov. 1, 1989 to May 31, 1993. The work was carried out at the Food Research Institute of University of Wisconsin-Madison under the direction of the principal investigator, Dr. F. S. Chu. The contract officer is Dr. Robert W. Wannemacher, Jr.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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ABSTRACT

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antibodies were obtained; (12) Several new immunogens, including hemiglutarate of decarbamoylated saxitoxin (DC-STX) conjugated to bovine serum albumin (BSA) and to keyhole limpet hemocyanin (KLH), carboxylmethoxyl derivative of STX (CMO) to BSA were prepared and their efficacy for antibody production were examined. These immunogens were not as effective as those prepared by Mannisch reaction where formaldehyde was used as the cross-linking reagent; (13) Pabs against neo-STX were produced and characterized. The antibodies not only are specific for neo-STX, but also have good cross-reactivity with STX. The ID $_{50}$ values for the binding of neo-STX-BSA to the anti-neo-STX-KLH by neo-STX, STX and DC-STX were 0.9, 8.0 and 53.1 ng/mL, respectively. However, the ID $_{50}$ values for the binding of anti-neo-STX-KLH to STX-polylysine coated on the microtiter plate by neo-STX, STX, and DC-STX were 1.2, 4.1 and 36.1 ng/mL, respectively. Using these antibodies, an effective ELISA for the determination of both STX and neo-STX was established; (14) Several hybridoma cell lines producing Mab against neo-STX were obtained. However, the affinity Mab to neo-STX and STX was much lower than Pab; (15) An improved procedure, including the use of immunoaffinity chromatography, for the isolation of STX and neo-STX from algal cells was established; (16) Five ml of anti-MCYST antiserum were delivered.

I. INTRODUCTION

Among many low molecular weight, heat resistant, naturally-occurring marine toxins, paralytic shellfish poisons and cyclic peptide toxins produced by algae are potentially hazardous to human health. Current analytical methods for monitoring these toxins in foods and in the environment are insensitive, non-specific and lengthy. No adequate diagnostic method is available for monitoring these toxins as well as their metabolites in human body fluids, tissues, and organs. In recognizing these problems, we proposed to develop sensitive diagnostic methods for selected dinoflagellate toxins such as saxitoxin (STX), and microcystin (MCYST), cyclic peptide produced by some cyanobacteria. The objectives of this project are two-fold: (A) development of methodologies for the production of different antibodies and different testing ligands, and different assay procedures for marine toxins; (B) production of specific immunoassay reagents for evaluation. To achieve these two objectives, the following work scopes were followed:

(1) development/improvement of methods for conjugation of two groups of marine toxins (STX and MCYST) to protein carriers;

(2) production of antibodies against the toxin-protein conjugates in rabbits;

(3) production of monoclonal antibodies against these two groups of toxins;

(4) improvement on immunoassay systems for the marine toxins described above and their application to military foods and human body fluids; studies such as the development of specific, sensitive and relatively simple *in vitro* methods for toxin detection (biosensor) other than RIA and ELISA will be included;

(5) immunoaffinity chromatography;

(6) isolation of STX receptor and development of antibodies against this receptor;

(7) exploratory studies on anti-idiotype (anti-Id) antibodies for STX and MCYST;

(8) collaborative studies with USAMRIID scientists on the following: (a) immunochemical studies on pharmacokinetics of the distribution of toxins in experimental animals; (b) immunohistochemical studies on STX and MCYST; and (ic) in vitro and in vivo testing for neutralization of STX and MCYST with antibodies;

(9) producting the following immunochemical reagents for evaluation: (a) specific radioactive markers, including tritiated saxitoxinol and reduced MCYST (10 mCi, at 5 Ci/mmole); (b) hapten-protein conjugate, antibodies and enzyme-linked toxin; (c) immunochemical reagents, which would be adequate for at least 10,000 assays of both toxins in the forms of ELISA; (d) reagents for other toxins will be supplied when they are available.

During the contract period, most of the proposed objectives were accomplished. Our work was concentrated in the following areas: (1) production of new immunogens for saxitoxin (STX) and neo-STX immunogens and subsequent testing of their efficacy for antibody production both in rabbits and mice; (2) establishment of an effective method for the production of polyclonal antibodies against neo-STX. The antibodies have shown cross-reactivity with both STX and neo-STX; (3) several hybridoma cell lines were established for the production of monoclonal antibodies (Mab) against MCYST; (4) several different STX or neo-STX conjugates as well as different immunization methods were tested for their ability to elicit antibodies against STX and neo-STX in BALB/c mice; one hybridoma cell line shown to produce Mab against neo-STX was obtained; (5) establishment of an ELISA method for monitoring of MCYST in blood and liver cytosol; this method has been shown to be very effective for monitoring the toxin in serum and liver cytosol after mice dosed with the toxin; (6) improvement of immunoassays: in this regard, immunoaffinity methods were used to purify marker ligands and antibodies; immunochromatography methods, including combination of TLC-ELISA, HPLC-ELISA and HPLC-RIA, were established for monitoring MCYST in feces and STX in algal cells; an improved indirect ELISA method was established for the analysis of STX and neo-STX by using different combinations of protein conjugates coupled with either neo-STX and STX; (7) critical evaluation of established immunoassays was made through analysis of various naturally occurring MCYST containing samples; (8) immunoaffinity methods were also established for the isolation of small amount of STX and neo-STX and for purification of tritiated marine toxins; (9) the effectivensss of antibodies in neutralizing the toxic effect of MCYST was tested by their effect in reversing the inhibitory of MCYST to protein phosphatase 2A *in vitro*. Various immunochemical reagents were prepared and evaluated in our laboratory through different studies; part of the reagents were delivered. Details of these studies as well as results obtained are described in the next section.

II. WORK PERFORMED DURING THE FIRST SIX QUARTERS

A. STUDIES ON MICROCYSTINS:

1. Production and characterization of polyclonal antibodies (Pab) against microcystin (MCYST):

a. Production of polyclonal antibodies: Routine production of Pab against

MCYST continued throughout the contract period. Rabbits previously immunized with MCYST leucine-arginine (LR) variant conjugated to polylysine and to EDA-modified BSA were boosted with the same immunogen once a month.

b. Further characterization of Pab: The cross-reactivity of MCYST-LR antibody with the newly isolated demethylated MCYST-LR (dm-MCYST LR), where the methyl group in the methylated aspartic residue in MCYST-LR is absent, was determined. The samples, which have been analyzed by HPLC method were supplied by Dr. Wayne Carmichael. ELISA analysis (MCYST-LR as the standard) revealed that the ELISA data were about 2.2 times more sensitive than the HPLC peak area for the dm-MCYST-LR than for the MCYST-LR. Thus, if both toxins have the same peak area at the same concentration, the antibodies would have higher affinity (2.2 times) for the dm-MCYST LR than for MCYST-LR itself.

2. Production and characterization of Mab against MCYST: Much of our effort during the contract was devoted to the production of Mab for MCYST. Intensive laborious screening of the clones was involved.

a. Production of monoclonal antibodies: Initially 6 BALB/c mice were immunized with MCYST-LR conjugated to EDA-BSA at a level of 20 µg each in the initial injection. Six booster injections, 20-30 µg each, were made at monthly intervals. Antibody titer was shown 12 weeks after immunization as determined by both an indirect ELISA and a radioimmunoassay (RIA). The spleen cells obtained from the mouse, which had the highest antibody titer, were fused with NS-1 myeloma cells. Subsequent screening, cloning and subcloning resulted a total of 10 stable hybridoma cell lines showing elicitation of antibodies against MCYST-LR as determined by both ELISA and RIA. Production of antibodies by 8 cell lines in ascites was made. Detail protocols and results obtained from each step of hybridoma cell screening, selecting and cloning were presented in our mid-term report.

b. Characterization of monoclonal antibodies against MCYST:

i. Preliminary characterization by RIA and recloning studies: The binding of tritiated MCYST with ascites fluid obtained from two of the above cell lines was studied by both equilibrium dialysis and ammonium sulfate precipitation methods. Either crude ascites fluid or ascites fluid purified by an ammonium sulfate precipitation method were used in the tests. Although competitive RIA revealed that the concentration causing 50% of inhibition of binding of tritiated MCYST-LR by unlabelled MCYST-LR was around 10 ng/assay (a level which is similar to the result when polyclonal antibodies were used), a high concentration of antibody was needed in the binding assay. Such results led us to perform another recloning study. However, we found that 5 of the 8 clones gave the same result as in the first cloning.

ii. Further characterization of monoclonal antibodies against microcystin (MCYST)-LR.

Monoclonal antibodies against MCYST-LR eliciting from 4 hybridoma cell lines (944F9C6, 944D4H1, 938H2G3 and 942C11B2) were prepared in ascites, purified by protein G affinity column chromatography and further characterized by an indirect competitive ELISA where MCYST-LY-polylysine was coated on the ELISA plate. All the Mabs are found to be in the subclass IgM and IgG1 type (1:1 ratio) with specificity for MCYST-LR. The concentrations causing 50% inhibition (ID_{50}) of the binding of Mabs to solid-phase MCYST-LR by MCYST-LR were in the range of 12 to 26 ng/mL. In contrast, the ID_{50} value of MCYST-LR for Pab was 2.2 ng/mL. The results indicate that the affinities of Mabs to MCYST-LR were about 10 times lower than the Pab.

For further characterization of Mab, a large amount of Mab from cell line 942C11B2 was produced. The efficiency with which the anti-MCYST-LR Mabs were recovered from the ascitic fluid by the ammonium sulfate (AS) precipitation and protein G affinity column was also compared. We found that the recovery of Mab by AS precipitation followed by protein G affinity column purification was low. Thus, for a long storage purpose, the Mab should be first purified by AS precipitation, dialyzed briefly and then lyophiled. Testing of the Mab from this cell line revealed that the Mabs are also cross-reactive with nodularin (NODLN), MCYST-tyrosine-arginine (MCYST-YR), but less reactive with MCYST-arginine-arginine (MCYST-RR) and MCYST-leucine-alanine (MCYST-LA). The ID_{50} values of the binding of Mabs to solid-phase MCYST-LR by NODLN, MCYST-YR, MCYST-LR, MCYST-RR, and MCYST-LA in the indirect ELISA were 3, 9.1, 16, 150, 933 ng/mL, respectively.

3. Establishment and improvement on the immunochemical analysis of microcystin (MCYST):

a. Establishment of an ELISA method for the analysis of MCYST in blood and liver cytosol:

A protocol for the analysis of MCYST in serum samples by ELISA was established. In the assay, one mL of serum sample (one mL) plus 5 mL of distilled water with pH adjusted to 3.0 was loaded to a C-18 Sep-Pak cartridge. After washing the cartridge with 25 mL of acidic distilled water (c.a. pH 3.0) followed by washing with 5 mL of 20% of methanol (MeOH), the cartridge was then eluted with 5 mL of various concentrations of aqueous methanol solution (60 to 100% MeOH). Among different methanolic solutions tested, 75% MeOH was found to be most effective for eluting MCYST from the cartridge with least interference substance in the sample. The solution eluted from the cartridge was then dried, reconstituted with phosphate buffer-saline (PBS) and subjected to ELISA. Recovery

experiments revealed that 68.5 and 104.5% of MCYST added to the serum at 1.0 and 100 ppb levels were detected by ELISA. Testing of different fractions of blood samples spiked with various amount of MCYST (0.2 to 100 ppb) reveal that good recovery (60 to 85%) was obtained at 1 and 100 ppb levels when either the serum or plasma were used. Storage of whole blood in the freezer before ELISA resulted in low analytical recovery. For best results, serum should be used in the analysis.

The same protocol was also shown to be very effective for analysis of MCYST in liver cytosol (1 mL of cytosol plus 5 mL of water with pH adjusted to 3.0 before subjected C-18 Sep-Pak cartridge). The recovery of MCYST added to the cytosol at levels of 10, 50 and 100 ppb was found to be 79, 97 and 99%, respectively.

b. Immunochromatography of MCYST: The effectiveness of the immunochromatography method (combination of ELISA and HPLC) for the identification of individual MCYST in feces of rats dosed with MCYST-LR and some of the naturally-occurring algal bloom samples was tested. Results of these studies are summarized as follows:

i. Identification of MCYST in rat feces by immunochromatography : Immunochromatography was used for further characterization of MCYST-LR in feces of rats that had been fed MCYST-LR. Extracts of rat feces provided by Dr. Wannemacher were first subjected to TLC (silica gel 60F 254 on a plastic backing, developed in a solvent system of chloroform/methanol/water at a ratio of 50/40/5). After separation, the TLC plate was cut into small pieces every 0.5 cm. Materials in each of these fractions were extracted with methanol and subjected to ELISA analysis. A typical immunochromatogram for sample 688-3, 2-24, is shown in Figure 1. In addition to MCYST (peak B), an immunoreactive derivative (peak A) which had a lower Rf value was found in all the samples tested. No immunoreactive peak was found in the control sample 688-3, 2-0. The concentration of MCYST and the new metabolites for four feces samples are given in Table 1. In general the total concentrations determined by immunochromatography, i. e. TLC and ELISA, are similar to those determined by ELISA without TLC.

ii. Identification of MCYST in liver cytosol: In a typical experiment, a cytosol solution containing about 252 ng of MCYST-LR was subjected HPLC. Almost 97% of the MCYST-LR as found by ELISA method before HPLC, was found by the HPLC-ELISA methods. It is interesting to note that a shoulder immunoreactive peak with apparent retention time of 13.5 min (one min. after MCYST) was found in the cytosol. The nature of this peak was unknown. Further identification of this minor peak is necessary.

iii. Identification of various MCYSTs in naturally occurring algal samples by immunochromatography:

The samples previously shown to contain MCYST by ELISA were subjected to HPLC followed by ELISA analysis. Results of a typical experiment for the analysis of a toxic algal bloom are shown in Fig. 2. It is apparent that more than one type cf MCYST are presented in the toxic algal bloom samples. MCYST-LR was found to be the major toxin involved in this bloom. Other MCYSTs, including RR, YR, and LA were also detected. Small amounts of different MCYSTs have also been found in the non-toxic blooms. Results of analysis of 26 naturally-occurring samples for MCYST by immunochromatography were presented in our 11th quarterly report. According to the distribution of different MCYST, the samples could be classified into 6 groups. MCYST-LR and MCYST-RR were the two major known MCYSTs that were most frequently found in these samples. It is interesting to note that 4 samples (2 from Lake Mendota of Madison, WI) have an unknown MCYST that was also reactive with MCYST antibodies. Further identification of this known is needed. Present data reiterated the analytical capacity and sensitivity of the immunochromatography method for the analysis of naturally occurring toxic samples if the antibodies used in the ELISA cross-react with structurally related toxins.

c. Immunoaffinity chromatography:

i. Purification of tritiated MCYST:

An antibody affinity column was prepared by conjugation of 31.2 mg of antibody to agarose gel (AminoLink gel, Pierce Chemical Co.). The column was equilibrated with 0.1 M sodium phosphate buffer containing 0.85% NaCL (PBS), pH 7.0. Tritiated MCYST-LR (10 uCi) in 1 mL of distilled water was added to the column. The column was then washed with 20 mL of distilled water, 14 mL of 60% ethanol and 5 mL of 100% ethanol, respectively. Immediately after elution with ethanol, the column was equilibrated with PBS containing 0.05% NaN3 and kept at 4C for repeated use. Most of the radioactivity (72.5%) was recovered in the 60% ethanolic fraction. ELISA analysis revealed that this fraction was reduced MCYST-LR and had a specific activity of 4,257 dpm/ng (1.92 Ci/mmole) compared to the original sample which had a specific activity of 945 dpm/ng (427 uCi/mmole). Approximately 25% of tritiated material was washed out by distilled water and showed no immunoreactivity with the antibody. A small amount of radioactivity (2.5%) was found in the 100% ethanolic fraction.

ii. Purification of antibodies against MCYST antibodies:

To improve immunoassay for MCYST, rabbit antibodies against MCYST were passed through an affinity column in which ethylene diamine modified BSA (EDA-BSA) was conjugated to Sepharose gel. Non-specific antibodies against EDA-BSA present in the antisera were removed by this treatment.

4. ELISA analysis of the kinetics of distribution of MCYST in serum and liver

cytosol after animal dosed with this toxin:

To test the effectiveness of the established ELISA protocol for analysis of MCYST in serum and cytosol, two experiments were made. One studies involved the analysis of a series of blood samples (supplied by Dr. Val R. Beasley of University of IL) from swine that had been dosed with toxic crude algal cells containing MCYST-LR. MCYST, at levels between 1 to 5 ppb, was found in the first 6 hours of serum samples. The levels after 6 hrs were less than 1 ppb, which is the detection limit for MCYST in serum by ELISA.

In the second experiment, the kinetics of distribution of the MCYST-LR in serum and liver cytosol of mice dosed with radioactive toxin (35 µg/kg) was analyzed by both radiotracer and ELISA methods. Result of this study is shown in Fig. 3. Detectable amounts of MCYST-LR were found in serum and liver as early as 15 min post-injection. MCYST-LR in serum peaked in 2 hr, then leveled off. The highest level of MCYST-LR was found in liver cytosol 12 hr post-injection. A considerable amount of toxin was still found in serum and cytosol at 24 hr post-injection. Similar data were obtained by the radiotracer method. An excellent correlation (r =0.99 at p < 0.0001) between data obtained from ELISA and radiotracer methods was found.

5. *In vitro* neutralization of MCYST toxicity by anti-MCYST antibodies:

Microcystins have been reported to be potent protein-phosphatase 1 and 2A (pp1 and pp2A) inhibitors both *in vitro* and *in vivo*. Recent studies have also demonstrated that MCYST-LR promoted tumor formation in rats. Because protein phosphorylation and dephosporylation play an important role in cell regulation, the cancer promoting activity of MCYST-LR in rats was attributed to its inhibitory effect on the phosphatase. To test whether the antibodies against MCYST can neutralize the toxicity, several *in vitro* experiments on the effect of MCYST-LR on the pp2A activity in the presence or absence of antibodies was carried out.

a. Preliminary study: In this study, MCYST-LR at a concentration of 10 µM was pre-incubated with the antibodies before running the pp2A assay. The results showed that pp2A activity was inhibited by MCYST-LR at a concentration as low as 1 µM. No such inhibitory effect was observed when 40 µL of rabbit anti-MCYST antiserum was preincubated with 10 µM of MCYST. Monoclonal antibodies from 4 hybridoma cell lines also reversed the inhibitory effect of MCYST for pp2A. Details for the preliminary study were presented in the 10th and 11th quarterly reports.

b. Quantitative assessment of reversal of the inhibitory effect of MCYST to pp2A by antibodies: Two approaches were made to determine the amount of ^{32}P incorporated into the protein. One approach involved counting the radioactivity in

the phospho-protein band after separation by SDS-PAGE. The other approach involved counting the radioactivity in a filter disc after separating the incubation mixture by membrane filtration. Both approaches yielded almost identical results. The results showed that MCYST is a very potent protein-phosphatase inhibitor; the incorporation of ^{32}P was completely inhibited by MCYST when only 30 pg of the toxin were present in the assay system. Polyclonal antibodies proved to be very effective in reversing such inhibitory effects and they are dose related. Results of a dose-dependent neutralization of the inhibitory effect of MCYST to PP2A by anti-MCYST polyclonal antibodies are shown in Fig. 4. Almost 80% of the enzyme activity was restored when as low as 0.012 µg of Pab was present. Monoclonal antibody was found to be least effective, as much as 48 µg Mab were needed in the assay system to achieve the same reversal effect by Pab.

c. Effect of anti-MCYST antibodies on the inhibitory effect of MCYST-LR, okadaic acid and calyculin A to pp2A: Results on the effects of okadaic acid, calyculin A and MCYST-LR on the catalytic activity of PP2A are compared in Fig. 5. Although calyculin A, okadaic acid and MCYST-LR are all potent inhibitors of phosphatase 2A activity, MCYST-LR was found to be 100 times stronger than calyculin A and okadaic acid for PP2A inhibition. To test if the anti-MCYST-LR Pab has cross reactivity to okadaic acid and calyculin A, Pab at a concentration of 48 µg/assay was preincubated with okadaic acid and calyculin A. The results showed that Pab has no effect on okadaic acid and calyculin A.

6. Production of anti-idiotype antibody against MCYST:

Attempts to produce anti-idiotype antibodies for MCYST were initiated. Two approaches were made. In the first approach, Mab against MCYST-LR obtained from cell line 942C11B2 was purified by a protein-G column and then immunized to three rabbits. However, no anti-idiotype antibody has been obtained 4 months after immunization and 3 booster injections.

In a second approach, polyclonal antibodies against MCYST-LR were subjected to an affinity column armed with MCYST-LR. The purified antibodies were then used as the immunogen for antibody production. Fig. 6 shows that anti-idiotype antibodies against anti-MCYST were produced in mice. In a competitive ELISA where anti-MCYST antibodies were coated in the ELISA plate, MCYST-LR could compete with the binding of the anti-idiotype antibodies to the solid-phase anti-MCYST antibodies (Fig. 7). The spleen cells obtained from one of these mice were used for the production of anti-idiotype monoclonal antibodies. Several hybridoma cell lines were obtained. Because of the end of this contract, the antibodies produced by these cell lines have not been characterized.

7. Other immunochemical studies on MCYST.

a. Analysis of MCYST in algae and in various samples collected from naturally occurring algal blooms: To test the effectiveness of the ELISA method developed in our laboratory for the analysis of MCYST, various samples of naturally occurring samples were subjected to ELISA. Detailed results of such analyses were presented in our mid-term report. The following conclusions could be made from some of these studies:

(i) The ELISA method could effectively be used for monitoring the toxins in the environment and could also be used for analysis of the toxins in water, algae and phytoplankton as well as in animal tissues and organs at a detection limit of 1 ppb except for water where as low as 2.3 ppt of MCYST-LR could be measured after concentrating the sample with a C-18 Sep-Pak cartridge.

(ii) The amount of MCYST in various toxic strains of algae appears to be very constant and generally in the range of 4 to 7 mg/gram of dry sample. Cells collected between 20 and 35 days contain more toxin (7 mg/g vs 4-5 mg/g) than those collected earlier or later.

(iii) MCYST levels in algae samples of naturally-occurring bloom samples varied considerably (ranged from 0.3 µg/g to 2610 µg/g). Low levels of MCYST were also found in a naturally-occurring neurotoxic field sample containing Anabaena (4 µg/g).

(vi) MCYST was concentrated in the hepatopancreas (HP) of freshwater clams. The concentration of MCYST in the HP (7.5 to 45 µg/g) was more than 10 times higher than in soft tissue (1 to 9.5 µg/g). Such data are consistent with those observed in other shellfish poisons.

(v) MCYST collected from the livers of ducks and fishes which died in algal blooms was minimal (0.03 to 0.6 µg/g). Thus, it is not known whether the death of the water fowl was due to MCYST intoxication.

(vi) MCYST concentration in samples collected from different depths of the lake were consistent with the level of phytoplankton in those samples. In general, surface samples contained higher concentrations of MCYST(30 µg/g in the surface vs 4.1 µg/g for samples at 1 meter and below). Since Oscillatoria were found in sediments, it is more likely that the toxin was MCYST-RR.

(vii) Correlation of the presence of MCYST in water of naturally-occurring algal blooms with the concentrations of MCYST in algal cells occurred only at low levels of MCYST in the cells (< 20 µg/g). It appears that there is an upper limit of MCYST, c.a. 100 ng/mL, present in the water under natural conditions.

b. Removal of naturally-occurring MCYST during water treatment: To test

whether the ELISA method could be used for monitoring MCYST in water supplies, a pilot study was carried out in collaboration with the California Dept. of Health. A limited water sample was collected from the Clear Lake area (CA) where a MCYST bloom occurred. Water samples (50 to 200 mL sample each) from the inlet and outlet area of several water treatment facilities were collected by scientists at the CDH. Each sample was loaded onto a Sep-Pak C-18 reversed phase cartridge and then sent to our laboratory. MCYST was eluted from the cartridge with 60% ethanol for ELISA. Among 5 sets of samples analyzed, the inlet level of MCYST ranged from 17.3 to 338 pg/mL (ppt) in comparison to the outlet level of 2.6 to 23 pg/mL (ppt). Preliminarily results indicated that 79 to 98% of the MCYST in the water supply was removed during regular municipal water treatment. The established method of collecting water and ELISA protocol certainly could be used to monitor trace amounts of MCYST in the water supply.

B. Studies on saxitoxin (STX) and neo-saxitoxin (neo-STX):

1. Production and characterization of antibody against STX by immunization of rabbits with decarbamoylated (DC) STX derivatives :

a. Preparation of decarbamoylated STX derivatives: Optimal conditions for the preparation of decarbamoylated STX (DC-STX), hemisuccinate (HS) DC-STX and hemiglutarate (HG) DC-STX were established. Analysis of DC-STX-HS and DC-STX-HG in solution revealed that DC-STX-HG was much more stable than DC-STX-HS. Thus, DC-HG-STX was conjugated to bovine serum albumin (BSA) and to keynole limpet hemocyanin (KLH), and horseradish peroxidase (HRP) using the water soluble carbodiimide method. Details for the preparation of these derivatives were presented in our mid-term report.

b. Development of an indirect ELISA for STX using anti-decarbamoylated STX antibodies: An indirect ELISA was developed for the determination of antibody titer of serum obtained from rabbits or mice which had been immunized with DC-STX derivatives. In the assay, DC-STX-HG-BSA/or DC-STX-KLH (100 ng/mL, 0.1 mL/well)) was coated to microtiter plates and a secondary IgG-HRP such as goat anti-mouse (or goat anti-rabbit) IgG-HRP conjugate, was used to detect the antibody bound to the solid-phase immunogen. The results showed that both conjugates show high binding capacity with anti-DC-STX-HS-BSA antibodies obtained from rabbits which had been immunized with DC-STX-HS-BSA.

b. Production and characterization of antibody against STX by immunization of rabbits with DC-STX-HS-BSA: The antibodies against STX were obtained after immunization of rabbits with DC-STX-HS-BSA. A radioimmunoassay (RIA) in which tritiated saxitoxinol (STXol) was used as the test ligand, was used for monitoring antibody production and characterization. Among three rabbits tested, only two rabbits showed good antibody titer. The concentrations causing 50% of inhibition

of binding of tritiated STXol by STX to anti-DC-STX-HS-BSA and to anti-STX-HCHO-BSA, i.e. antibodies obtained from rabbits that have been immunized with STX-HCHO-BSA prepared by the formaldehyde method, were found to be 11.8 and 5 ng/mL, respectively. Thus, the affinity of STX to the anti-DC-STX-HS-BSA antibody was about one-fold less than anti-STX-HCHO-BSA antibody. The results indicated that DC-STX-HS-BSA could be used as an alternative immunogen for the preparation of antibody against STX.

c. Production of antibody against STX by immunization of rabbits with DC-STX-HG-KLH: Three rabbits were immunized with 0.5 mg of DC-STX-HG-KLH each by a multiple injection method and were boosted with the same antigen every four weeks. The indirect ELISA described above was used to monitor the antibody titers. The titers for a nine week bleeding were about 2000. When 100 ng/mL of saxitoxin was added in the assay, the binding decreased to 67%. The titer was considerably lower than the antiserum obtained from a rabbit (DSB-1) which had been immunized with DC-STX-HS. The antibody titer of DSB-1 was found to be approximately 60,000 under the same immunoassay conditions. However, the anti-DC-STX-HG-KLH antibody is more sensitive for use in the indirect immunoassay. For example, the binding only decreased to 89% after addition of 100 ng/mL of saxitoxin in the indirect ELISA assay, when DSB-1 antibody was used.

2. Production of antibodies against STX by using carboxymethoxyl-derivative of STX (STX-CMO):

Carboxymethoxy-STX was prepared under the conditions described in our mid-term report and then conjugated to KLH and BSA via a water soluble carbodiimide method. Three rabbits were immunized with 0.5 mg of STX-CMO-KLH in complete Freund's adjuvant and boosted with 0.5 mg of the same antigen in incomplete Freund's adjuvant every four weeks thereafter. Five weeks after the first injection, the titers of antisera had already reached 10,000. However, there was no displacement when saxitoxin standards were added. The study was not continued.

3. Production and characterization of antibody against neo-STX by immunization of rabbits with neo-STX-KLH and neo-STX-BSA: Antibody against neo-STX was obtained nine weeks after immunization of rabbits with neo-STX conjugated to either KLH or BSA via the Mannisch reaction under the conditions similar to those for the conjugation of STX to proteins. Formaldehyde was used as the cross-linking reagent. An indirect ELISA, in which either neo-STX-BSA or neo-STX-KLH was used as the coating reagent, was used to monitor the antibody titer and to characterize the antibody. Although high antibody titers were obtained from rabbits after immunization with both immunogens, only antibody obtained from rabbits immunized with neo-STX-KLH were useful for immunoassay.

Competitive indirect ELISA revealed that the antibodies obtained from rabbits immunized with neo-STX-KLH are specific for neo-STX, but also have good cross-reactivity with STX. The concentrations causing 50% inhibition binding of neo-STX-BSA to the anti-neo-KLH by neo-STX, STX and decarbamoyl-STX (DC-STX) were 0.9, 8.0 and 53.1 ng/mL, respectively. Saxitoxin conjugated to polylysine (STX-PLL) was also used as the coating reagent in the indirect ELISA. The concentrations causing 50% inhibition binding of anti-neo-STX-KLH to STX-PLL coated on the microtiter plate by neo-STX, STX, and DC-STX were 1.2, 4.1 and 36.1 ng/mL, respectively. Using this newly developed antibody, ELISA could be very a effective method for monitoring seafood for both neo-STX and STX.

The antibody appeared to have only weak cross-reaction with saxitoxinol (STXol). A high concentration of antiserum (one to 20 dilution) was necessary to give 50% binding of 10,000 dpm of tritiated STXol in the assay system. Competitive RIA for monitoring of STX and neo-STX was also less sensitive when anti-neo-STX and tritiated STXol were used. The concentration causing 50% inhibition of binding of tritiated STXol to the antibody by STX and neo-STX in RIA was found to be around 22 and 58 ng/mL, respectively.

Details for the production and characterization of the antibodies against neo-STX were published in the J. AOAC. (Publication no. 9).

4. Production of monoclonal antibodies against neo-STX:

Several attempts to produce Mabs against STX or neo-STX were made. Initially, several groups of mice were immunized with DC-STX-HG-KLH and DC-STX-HG-BSA. Although antibodies against the conjugates were demonstrated, ELISA analysis revealed that the antisera had low affinity to free STX or neo-STX (see mid-term report). In view of our success in obtaining good Pab against neo-STX by immunization of rabbits with neo-STX conjugated to either KLH or BSA, several approaches attempting to produce monoclonal antibodies against neo-STX were made.

a. Monoclonal antibodies against neo-STX by fusing NS-1 myeloma cells with spleen cells of BALB/c mice that had been immunized with neo-STX-KLH conjugate: Our first attempt involved fusing of the spleen cells obtained from a mouse that had been immunized with neo-STX-KLH with highest antibody titer against neo-STX with NS-1 myeloma cells. Details of this study were reported in our mid-term and 7-9th quarterly reports. In this study, four master cell lines elicited antibodies that were capable binding to STX-polylysine with the binding being displaced by neo-STX. However, only one master cell line was stable. Subsequent cloning and subcloning of this stable master cell line led to 4-5 hybridoma cell lines that elicited antibodies against Neo-STX and STX. Further purification and characterization of antibodies revealed that the antibodies belong

to IgG1, κ type. However, we found that the affinity of the antibody was low; a large amount of Mab was necessary for the immunoassay of Neo-STX.

b. Production of Mab against neo-STX involving use of new conjugates and new immunization schedule: Since the affinity of the Mab produced by the above cell lines to neo-STX was low, the immunogenicity of 3 new conjugates was tested for the production of antibodies against neo-STX in BALB/c mice. Neo-STX conjugated to staphylococcal enterotoxin B (SEB) and KLH showed better performance than neo-STX conjugated to BSA. Highest antibody titers were obtained in mice immunized with neo-STX-KLH together with SEB. The mouse polyclonal antibodies also have been very effective in a competitive ELISA (Fig. 8). The sensitivity of this ELISA for neo-STX detection is comparable to the rabbit antiserum. This result indicates that high affinity antibodies against neo-STX have been generated. Consequently, spleen cells from this group of mice were used for fusing, cloning and subcloning. A hybridoma cell line (2D2) eliciting Mab (IgM type, kappa) against neo-STX was obtained.

5. Improvement of ELISA sensitivity for STX and neo-STX analysis:

a. Improvement via use of purified antibody preparations: The first approach that we tested to improve the indirect ELISA of STX involved the use of purified antibodies. Antiserum against STX, which was prepared by ammonium sulfate precipitation, was passed through an affinity column to remove non-specific antibodies against BSA and modified BSA. Results showed that antibodies after such treatment provided a steeper displacement curve than those without treatment.

b. Improvement via using neo-STX-protein conjugates: With availability of pure neo-STX standard supplied by Dr. Sherwood Hall, we have re-evaluated the cross-reactivity of the anti-STX antibodies prepared in our laboratory with STX and neo-STX. We found that the affinity of the antibodies to neo-STX is almost 100 times less than that of STX, in RIA. Based on this observation, we rationalized that if neo-STX-protein conjugate is coated on the microtiter plate for ELISA, much less STX would be needed for competition of the binding of the antibody with solid-phase antigen. Consequently, neo-STX was linked to BSA and to KLH in the presence of HCHO. Both neo-STX-HCHO-BSA and neo-STX-HCHO-KLH were then used in indirect ELISA for STX analysis. Results of these studies are shown in figure 9. When neo-STX-BSA was used, the concentrations causing 50% inhibition of binding of antibody to the solid-phase antigen by STX and neo-STX were found to be 1.1 and 44.7 ng/mL, respectively. With neo-STX-KLH, the concentrations causing 50% inhibition of binding of antibody to the solid-phase antigen by STX and neo-STX were found 0.23 and 18 ng/mL, respectively. Thus, the present system is about 3-10 times more sensitive than the one we reported before, when STX-HCHO-polylysine was used to coat the plate. From the competition indirect

ELISA curves, it is apparent that the cross-reactivities of the antibodies with STX in the solid-phase is 40-80 times than more with neo-STX.

6. Affinity chromatography of STX using antibodies specific for STX:

a. Preparation of affinity columns: Two types of affinity columns with different STX binding capacities were prepared. The low capacity column (0.2 to 0.3 ug STX/column) was prepared by conjugation of one mL of anti-STX antiserum (ammonium sulfate precipitation cut of antiserum batch 15#35N), which contained of 29 mg of protein, to 2 mL of AminoLink gel (Pierce Co.) using the reductive alkylation method.

For the high capacity column (0.7 to 0.8 ug STX/column), purified antibody was used. The anti-STX antiserum was first purified by passing through a AminoLink column coupled with BSA, which had been previously treated with HCHO. The non-specific antibodies against HCHO-BSA and BSA were removed by this treatment. In a typical experiment, 43.3 mg of the purified antibodies (equivalent to 5.9 mL of anti-STX antiserum 15#31N and 40N) were conjugated to the 2 mL AminoLink column.

b. Conditions for separation of STX and neo-STX by affinity chromatography: In general, one mL of sample (pH 7.5) containing less than 200 ng (low capacity column) or 700 ng (high capacity) of STX, was loaded onto the column. Two tenth (0.2) mL of 0.01M phosphate-saline buffer (PBS, pH 7.5) was then added. After incubation at room temperature for one hr, the column was eluted with either 10 mL of 0.2M glycine-HCl buffer (pH 2.65) or washed with 5 mL of 0.001 M acetic acid (HoAc) to elute neo-STX and then followed by 5 mL of 0.2 M glycine-HCl buffer (pH 2.65) to elute STX. The column after elution with glycine buffer was re-equilibrated with 20 mL of 0.01M PBS immediately. For storage, the column was washed with 0.05% sodium azide solution and kept at 4C. Before reuse, the column was then washed with 20 mL of 0.01M PBS. Details of various studies were described in the 5th quarterly report.

c. Results obtained: Various studies on the affinity column chromatography of STX have led to the following conclusion: (i) the affinity column is very effective for trapping STX but not for neo-STX; (ii) the column can be used as a clean-up tool for STX analysis; (iii) the column can be used for isolation of a small amount of STX; combination of mouse assay and RIA showed that STX eluted from the column has a constant ratio of STX (determined by RIA) and mouse units (between 180-200 ng/mouse unit); (iv) if a sample contains only STX and neo-STX, the column could be used to purify these two toxins; (v) the capacity of the column for STX should be pre-determined for quantitative purposes; (vi) higher capacity column could be used for preparative purposes; and (vii) the column could be used repeatedly many times after regeneration.

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7. Isolation and purification of neosaxitoxin from the culture of _Aphanizomenon_
flos-aguae NH-5. Because antibodies against STX had little cross-reactivity with
neosaxitoxin (neo-STX), attempts to isolate a small amount of neo-STX were made.
Aphanizomenon flos-aguae NH-5 cells containing both neo-STX and STX were
obtained from Dr. Wayne Carmichael's laboratory. The procedure developed by Dr.
Carmichael's Lab. (Toxicon 24: 175-186) was used with some modifications in the
chromatography step. An immunoaffinity column (2 mL size), which has a capacity
of 700 ng of STX (or 3-4 mouse units), was used as the last step for separation of
neo-STX and STX. Radioimmuncassay and TLC, as well as mouse assay were
used to follow each of the following purification steps. Detailed protocols for
various purification steps were summarized in the 5th quarterly report.

Results of the preparation of neo-STX and STX from one gram of
Aphanizomenon flos-aguae NH-5-a cell are presented in Table 5. Since the affinity
of the antibody used in the RIA is almost 100 times higher to STX than that of
neo-STX, the ratio of STX concentration as determined by RIA to the mouse unit
was used as an index for the separation of neo-STX and STX. A typical elution
pattern, as determined by both RIA and mouse assay for the CM-sephadex step is
shown in Figure 10. It is apparent that the majority of neo-STX was eluted from
the column in the 0.2 M HoAc fraction where the ratio of STX to mouse unit was
around 50. However, STX was eluted in the 0.25 and 0.3 M HoAc fractions.

From Table 2, it is apparent that whereas a large amount of contaminating
materials were removed in the first three steps, no separation of STX and neo-STX
was achieved. A slight separation of neo-STX and STX could be seen in the CM-
Sephadex chromatography step. Final separation of neo-STX and STX can only be
achieved after affinity column chromatography. The final preparation for STX (5B)
has a mass unit consistent with mouse unit of the reported data (180 ng/mu).
However, the ratio of STX/MU for the 5A fraction, which is considered to be neo-
STX, was 14.5. The lowest ratio of STX/MU for 5A fraction obtained from several
other affinity chromatograph runs was 8.2. These values are not consistent with
the theoretical value of 1.82-2.0, which is estimated from the cross-reactivity of
the antibody with STX and neo-STX (50 to 100 times more for STX than for neo-
STX). Nevertheless, a very minor contamination of STX (1%) in the preparation in
combination with the uncertainty of a quantitative value for neo-STX and problems
in the accuracy of the mouse assay, could all lead to a higher experimental ratio of
STX/MU. Thin layer chromatography analysis of 5A fraction revealed a single spot,
which was identified as neo-STX.

III. DELIVERABLES

Five ml of anti-MCYST-LR antiserum were delivered during the year.

IV. DISCUSSION

In the present project, our research was focused on three general areas, namely: (1) development of new and better methods for production specific antibodies against two major groups of marine toxins; (2) establishment of new and improvement of existing immunoassay protocols; and (3) testing the possibility of using antibodies of as a therapeutic agent. Throughout these investigations we also evaluated effectiveness of the reagents prepared through various immunochemical analyses. The progress in each of these areas are assessed in the following discussion.

Several different new approaches for the production of antibodies against STX and neo-STX were established. Whereas antibodies were obtained from rabbits after immunization with DC-STX-HS-BSA, the affinity of the antibody to STX was not as good as those immunized with STX-HCHO-BSA. Unfortunately, these antibodies cross-reacted with neo-STX weakly. Such data led to our decision to include the development of antibodies against neo-STX. With the supply of a larger amount of neo-STX from Dr. Hall of FDA, several neo-STX protein conjugates were prepared for various studies. Comparing with two different cross-linking reagents used, we found that formaldehyde was a better cross-linking reagent than glutaraldehyde because the latter reagent caused protein aggregation. We have successfully obtained polyclonal antibodies that have good cross-reactivity to both neo-STX and STX. Such antibodies will be very useful for both analytical and therapeutic proposes. An indirect ELISA, in which either neo-STX-BSA or STX-PLL could be used as the coating reagent, was established for simultaneous analysis of neo-STX and STX.

Effort for the production of monoclonal antibodies against MCYST and neo-STX were made. Eight hybridoma cell lines eliciting monoclonal antibodies against MCYST-LR were obtained. Since the antibodies belong to a mixture of IgG and IgM1 (1:1 ratio), these antibodies tend to aggregate in solution. The affinity of the Mab to MCYST-LR was found to be 10 times less than Pab. Such data suggest that the Mab is less effective for immunoassay, but it could be used as the affinity reagent for the preparation of antibody based-affinity column.

Since monoclonal antibodies against STX have been obtained by Dr. Chanh's group by using HCHO-STX (Huot et al., 1989; J. Clinical Invest. 83: 1821-1826) as well as by a Canadian group, our effort toward the production of monoclonal antibodies for STX was concentrated on other immunogens. Thus, production of antibodies against STX and neo-STX in mice by several new immunogens (such as STX-enterotoxin B conjugate) was compared. Neo-STX-HCHO-KLH was found to be the most effect effective immunogen when immunized together with staphylococcal enterotoxin B. Polyclonal antibodies obtained from mice immunized with this immunogen by this approach also shown to be very effective in an indirect ELISA for neo-STX. A hybridoma cell line eliciting Mab against neo-STX was also obtained after two extensive fusings and clonings. This Mab belongs to

the IgM, k and has an apparent affinity to neo-STX about 100 times less than the Pab, as judging from the competitive indirect ELISA. Further characterization of this Mab is needed.

Regarding the improvement and development of new analytical methods, we have established an effective ELISA protocol for the analysis of MCYST in serum, urine, cytosol, animal tissues, organs in addition to algal cell and water. Kinetics analyses of the distribution of MCYST in serum and liver cytosol of mice receiving the toxin clearly demonstrated the effectiveness of this method. Analysis of various field samples containing naturally occurring MCYST also showed that ELISA is an effective method and can detect trace amounts of MCYST in the environment. For example, as low as 2.6 pg of MCYST could readily be determined in water (2.6 ppt). Most of the MCYST in the water supply could be removed by regular municipal water treatment. Our data also showed that treatment of water with C-18 reversed phase Sep-Pak cartridge could be an efficient method for adsorption (or removal) of MCYST. Affinity chromatography has been used extensively in various investigations in this contract. Both RIA and ELISA improved considerably after using the affinity purified antibodies and radioactive markers. Improvement of ELISA could also made by using different coating immunogens; for example, we found that the sensitivity of ELISA for STX improved 10 times when neo-STX-KLH was coated in the ELISA plate.

Immunochromatography has been found to be very useful both on an analytical scale and for preparative scale. For example, combination of TLC with ELISA was used in the identification of immunoreactive compounds in fecal samples of rats that had been fed with MCYST as well as for the identification of various MCYST in algal samples collected from toxic blooms. Combination of RIA with liquid chromatography was used to monitor the toxin containing fractions in the preparation of STX and neo-STX.

Regarding the possibility of using antibody as a prophylactic agent, we found that Pab against MCYST effectively neutralized the inhibitory effect of the toxin to pp2A activity. Mab was found to be less effective than the Pab for this reversal effect. The data are consistent with the finding that the affinity of Mab to MCYST is less than Pab. We also found that the antibodies have no effect on reversing the inhibitory effect on pp2A caused by okadaic acid and calyculin A. These data suggest that similar epitopes in the MCYST-LR molecule might be involved in binding to the antibody as well as to the pp2A. It is also possible that anti-MCYST-LR antibodies block the inhibition ability of MCYST-LR to pp2A by changing the toxin's conformation. The data further reiterate the specificity of anti-MCYST Pab to MCYST-LR. Thus, it is possible to identify MCYST-LR related compounds in a naturally-occurring sample that has been shown to have an inhibitory effect to PP2A when the samples were preincubated with the anti-MCYST antibody. This approach could also be used to distinguish the inhibitory

effect of MCYST on pp2A from other environmental toxicants. The overall results suggest that it is possible to use antibodies to neutralize MCYST toxicity.

Preliminary studies revealed that anti-idiotype antibodies against anti-MCYST antibodies were obtained. We have demonstrated that these antibodies could mimic MCYST in a direct ELISA. Whether this anti-idiotype antibody could be used as a therapeutic agent remains to be studied.

In conclusion, whereas we have achieved most of our objectives, much related work still needs to be done, especially in the generating of Mab and anti-idiotype antibody areas. The affinity of the Mabs obtained from our laboratory as well as others to STX and MCYST related toxins are lower than Pab. To overcome this problem, genetic engineering of the existing hybridoma cell lines producing such antibodies should be done. Through an understanding of the epitopes involved in the binding of Ig to the ligand as well as the cDNA structure, it is possible to engineer the specific antibodies for these toxins. Likewise, point mutation could be used. Since good Pabs against both toxins are available, generating effective anti-idiotype antibodies is another effective approach. Our data on MCYST clearly demonstrated such feasibility.

V. PUBLICATIONS

A. Manuscript issued:

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B. Manuscript in preparation:

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2. Lin, I. and Chu, F. S. (1993) Kinetics of distribution of microcystin-LR in serum and liver cytosol of mice dosed with the toxin: An immunochemical analysis (in preparation).

3. Lin, I. and Chu, F. S. (1993) Reversal of inhibitory effect of microcystin-LR to protein phosphatase 2A activity by antibodies (In preparation).

4. Chu, F. S., Hsu, K. H., Huang, X. and Lin, I. (1993) Production and characterization of monoclonal antibodies against microcystin-LR (in preparation).

5. Huang, X. and Chu, F. S. (1993) Production and characterization of monoclonal antibodies against neo-saxitoxin (in preparation).

Table 1. Analysis of MCYST in rat feces by
ELISA and immunochromatography

Sample No.		Toxin concentration (ng/mL)		
	ELISA	TLC-ELISA		
		Peak A	Peak B[a]	Total
688-3, 2-0	0	0	0	0
688-3, 2-24	560	239	220.3	459.3
688-3, 3-24	81	220.8	169.7	390.5
688-3, 4-24	940	280.8	635.8	916.6
688-3, 7-24	500	190.1	452.7	642.8

[a] identified as MCYST

Table 2. Isolation of STX and neo-STX from
Aphanizomenon flos-aquae NH-5

Purification Steps	Yield (mg)	MU	STX (RIA) (µg)	STX/MU (ng/MU)
1. Cell Extract	1000	2163(100)[a]	220 (100)	101.7
2. Ethanolic Extract	600	2031 (93.9)	124 (56)	61.0
3. P-2 Gel filtration	51	1904 (88)	118 (54.6)	61.0
4. CM-Sephadex				
A'			0.8	
A		405	21.7	54.0
B		135	11.6	85.9
C		225	27.0	120.0
D		96	15.4	160.4
5. Affinity column [b]				
A		2.2	32.9	14.9
B		2.0	365.0	182.5

[a] Values in parenthesis represent % of recovery.
[b] Equivalent to 414 ng (60 ng/MU) of STX obtained from step 4-A
were loaded onto the column.

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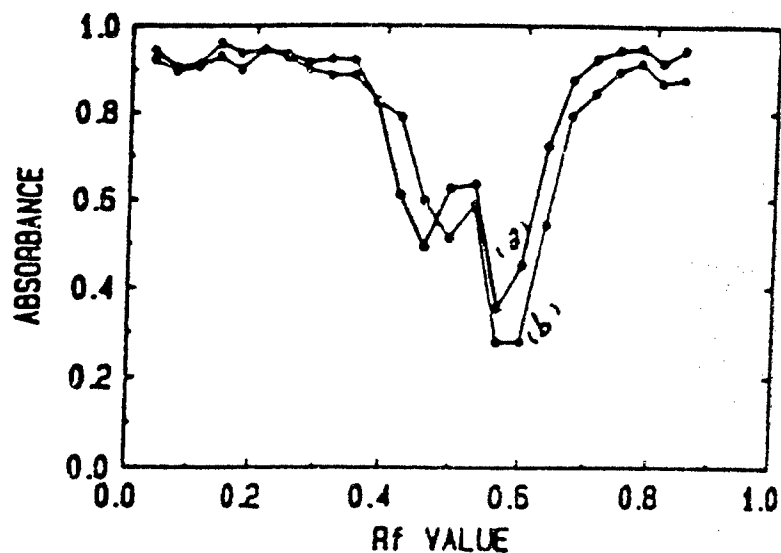
Figure 1. Immunochromatogram of rat feces extract for microcystin-LR. Chromatogram a (open circles) and b (solid circles) on this figure represent two samples, one spotted with 0.15 ml of 2-24 sample extract and the other spotted with same amount of 2-24 extract together with 80 ng of MCYST. The higher Rf spot was identified as MCYST.
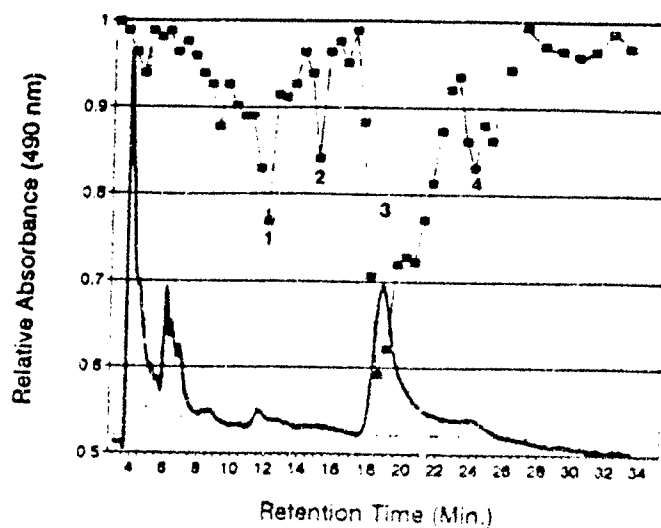

Figure 2. Immunochromatography of MCYST. Regular HPLC elution pattern and ELISA chromatogram are shown in the bottom (positive peaks) and top part (negative peaks) of the figure, respectively. Peaks 1, 2, 3, 4 correspond to the retention of MCYST RR, YR, LR and LA, respectively.
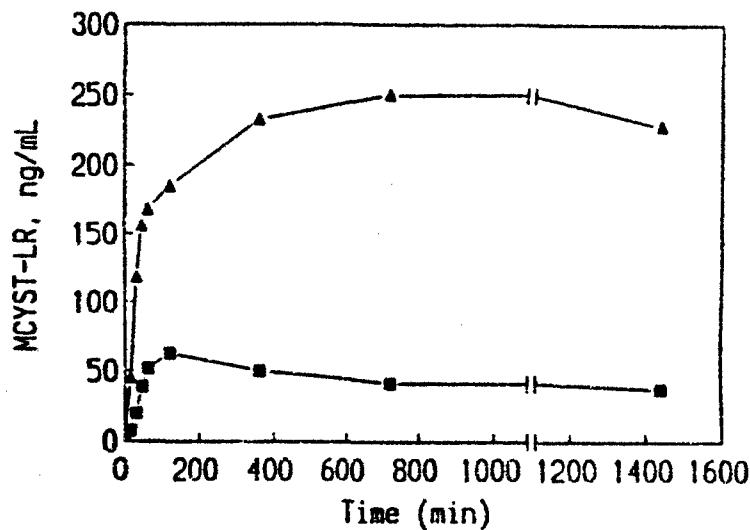
Figure 3. Kinetics of appearance of MCYST-LR in mouse serum and liver cytosol. Mouse serum and liver cytosol samples were analyzed for MCYST-LR with a direct competitive ELISA after C-18 reversed phase cartridge cleanup. The concentrations of samples at each time point were plotted as ng/ml vs. time. The square and triangle represent the serum (■) and liver cytosol (▲), respectively.
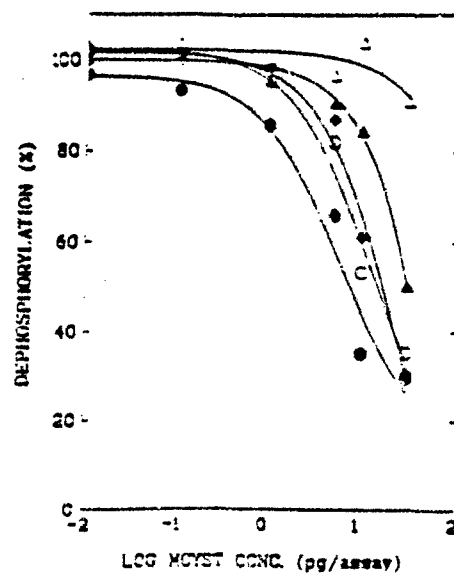
Figure 4. Dose-dependent neutralization of the inhibitory effect of MCYST to protein phosphatase by anti-MCYST-LR polyclonal antibody. Various concentrations of MCYST-LR (0.01 pg/assay-30 pg/assay) and polyclonal anti-MCYST-Pab (0.00012 μg/assay-1.2μg/assay) were incubated with a PP2A/32P-histone H1 (0.4mU/10 μg) mixture in room temperature for 1 hr. The radioactivity in the phosphoprotein was determined after separation of the phosphoprotein by SDS-PAGE and slicing the gel for radioactivity profile. Following symbols represent different antibody dilution: 1.2 μg/assay (▲), 0.12 μg/assay (▲), 0.012 μg/assay (♦), 0.0012 μg/assay (○) and 0.00012 μg/assay (●).
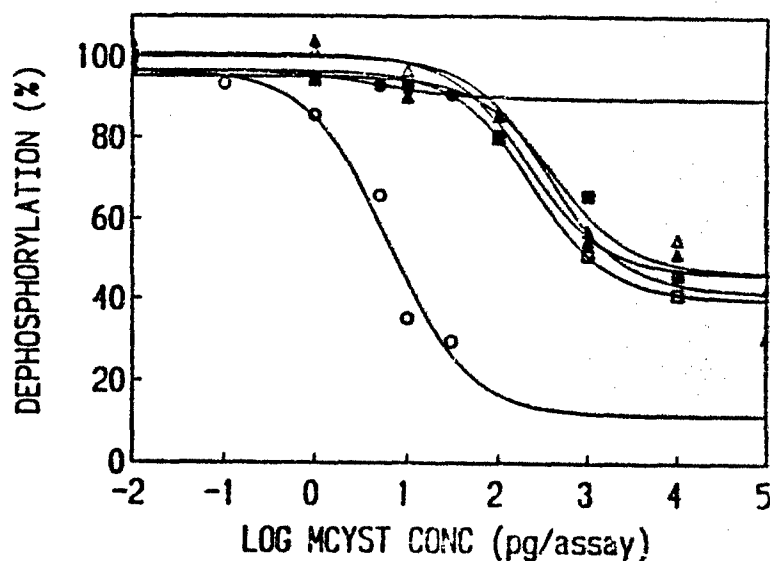
Figure 5. Effect of anti-MCYST polyclonal antibody on the inhibition of Microcystin-LR, okadaic acid and calyculin A to PP2A. Various concentrations of MCYST-LR (0.01-30 pg/60 μL assay), okadaic acid and calyculin A (0.001-100 ng/assay) in the presence and absence of anti-MCYST Pab (10 μg/assay) were incubated with PP2A(0.4mU)/^{32}P Histone H1 (1 μg) mixture at room temperature for 1 hour. The open circles, triangles and squares represent MCYST-LR (O), okadaic acid (Δ) and calyculin A (□) in the absence of antibody, and closed circles, triangles and squares represent MCYST-LR (●), okadaic acid (▲) and calyculin A (■) in the presence of Pab.
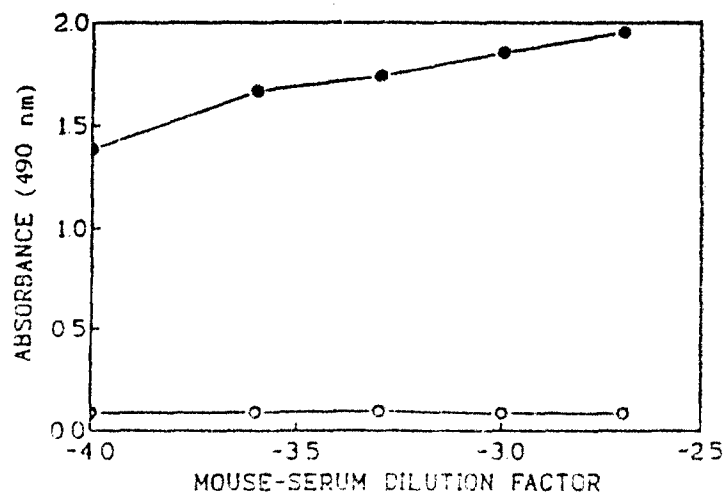
Figure 6. Production of anti-idiotype antibodies in mouse after immunization with affinity purified rabbit anti-MCYST antibodies. The ELISA plate was coated with rabbit anti-MCYST Pab at a dilution of 1:1000. (●, 4 weeks bleeding; O, preimmune serum)
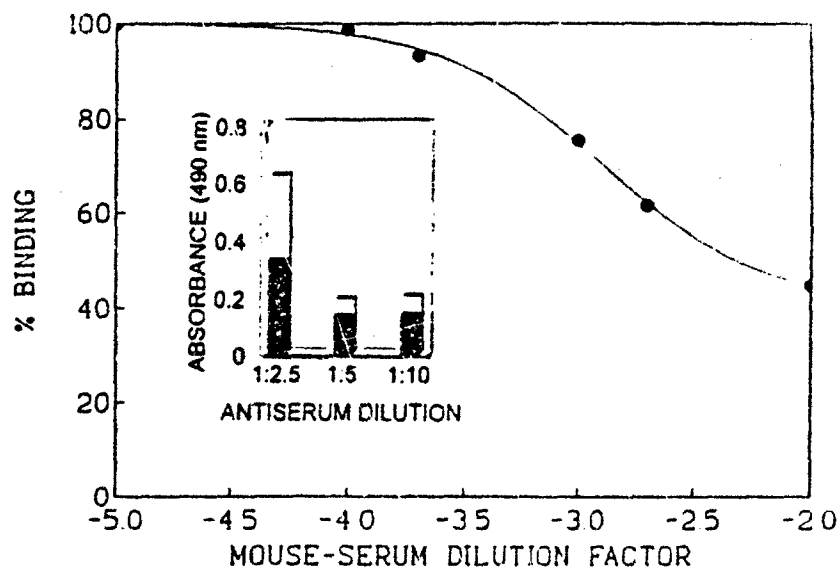
Figure 7. Competitive ELISA for the analysis of anti-idiotype antibodies against rabbit anti-MCYST Pab. The ELISA plate was coated with anti-MCYST Pab (1:1000 dilution) and MCYST-HRP (1:800 dilution) was used as the marker enzyme conjugate. The insert represents the analysis of the supernatant fluid obtained from a hybridoma cell line 8G4 using the methods as described in fig. 6; solid bars represent data from the ELISA containing 1 µg of MCYST.
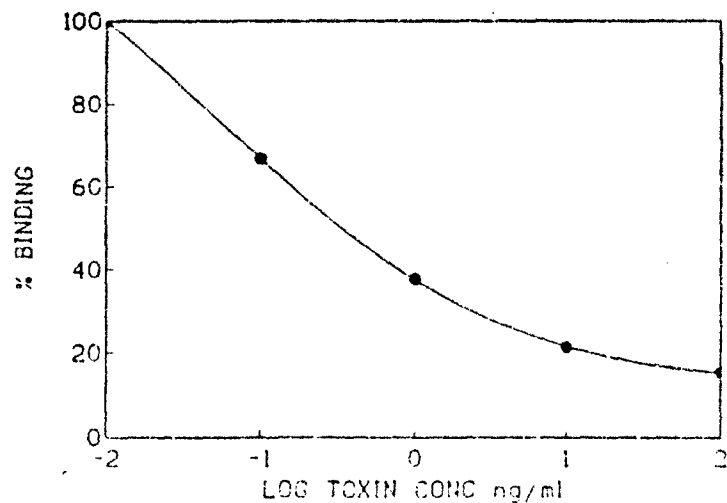

Fig. 8. Competitive ELISA for neo-STX using polyclonal antibodies obtained from a BALB/c mouse that had been immunized with neo-STX-KLH together with SEB. The antiserum dilution was 1:30 000. Neo-STX-polylysine at a concentration of 2 µg/mL was coated to the ELISA plate.
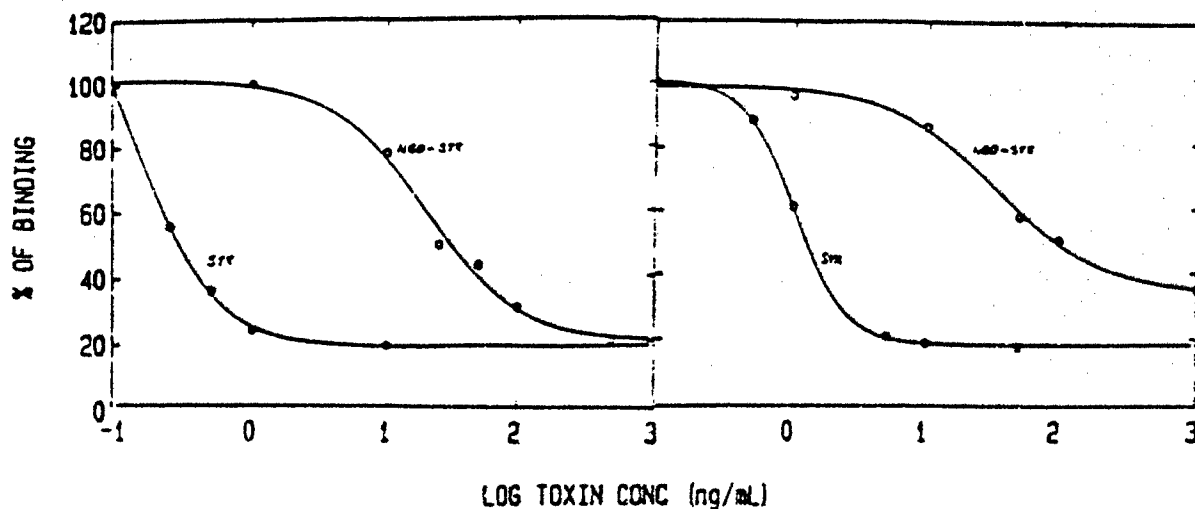
Figure 9. Competitive indirect ELISA of STX and neo-STX. neo-STX-HCHO-KLH (left) and neo-STX-HCHO-BSA (right) were used as the coating immunogens in these assays.
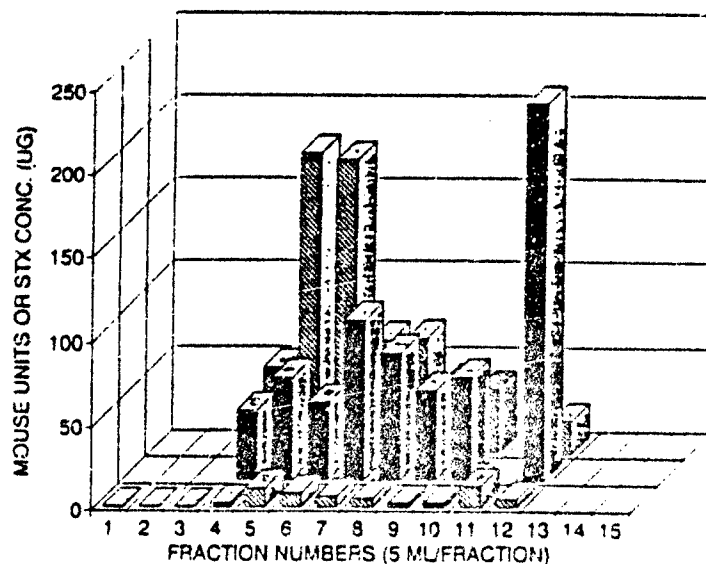

Figure 10. Immunochromatogram of STX of partially purified STX and neo-STX on a CM Sephadex column. Samples obtained from Bio Gel P-2 column were applied to the column followed by elution with various concentrations of HoAc under the conditions described in the text. The elution pattern was obtained by analysis of various fractions for STX (RIA) and mouse potency after elution with 0.2M HoAc. The column was eluted with 0.25 M HoAc and 0.3 M HoAc at fraction number 9 and 15, respectively. Data in the front, middle and back are STX concentrations (μg/tube) determined by RIA, STX/mouse unit (ng/mouse unit) and mouse units, respectively.